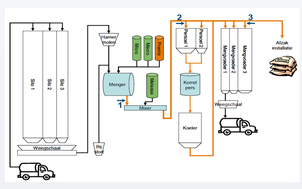
Concentrations of selected antimicrobials in caecum, colon and manure of pigs due to a 3 % cross-contamination of the feed and their effect on resistance in *Escherichia coli*

Peeters L[1,2], Smet A[2], Daeseleire E[4], Devreese M[3], Dewulf J[5], Heyndrickx M[4], Haesebrouck F[2], Croubels S[3], *Butaye P [2, 6]

[1] Department of General Bacteriology, Veterinary and Agrochemical Research centre, Brussels, Belgium; [2] Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Merelbeke, Belgium; [3] Department of Pharmacology, Toxicology and Biochemistry, Ghent University, Merelbeke, Belgium; [4] Technology and Food Science Unit, Institute for Agricultural and Fisheries Research, Melle, Belgium; [5] Department of Reproduction, Obstetrics and Herd health, Ghent University, Merelbeke, Belgium; [6] Department of Biosciences, Ross University, St Kitts, West Indies

Introduction



3%

Pig feed may contain up to 3% carry-over of the recommended therapeutic concentrations of antimicrobials[1] as a result of contamination between medicated and non-medicated feed. The gut concentrations of three commercially available formulations of antimicrobials, frequently used in pig rearing, were determined through an *in vivo* experiment in order to investigate their possible selective effect for resistant bacteria. The effect of 1 µg/ml and 4µg/ml doxycycline on resistance selection and transfer in *E. coli* was investigated *in vitro*.

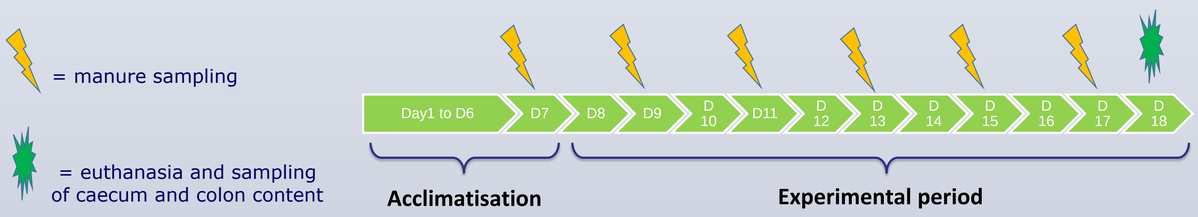
[1]Based on the EU guideline regarding coccidiostats or histomonostats (2009/8/EG). Since 2013, limits of 1% of minimal therapeutic concentrations are applied in Belgium.

Materials and methods

1. Feed: 3 batches with a 3% carry-over level of the recommended therapeutic dose of **doxycycline (DOX)**, **sulfadiazine-trimethoprim (SULFA-TRIM)** and **chlortetracycline (CTC)**, respectively.

2. Animal experiment

Twenty-four pigs were equally divided into one control group and three experimental groups receiving experimental feed during 10 days.



3. Chemical analysis

Antimicrobial concentrations were determined using in-house developed and validated LC-MS/MS methods.

4. Based on the animal experiment results (see below), we decided to investigate the selective effect of **4 µg/ml DOX** (corresponding to 3% cross-contamination of the feed) and **1 µg/ml DOX** (1% cross-contamination) *in vitro* in selected *E. coli* isolates.

5. Bacterial strains

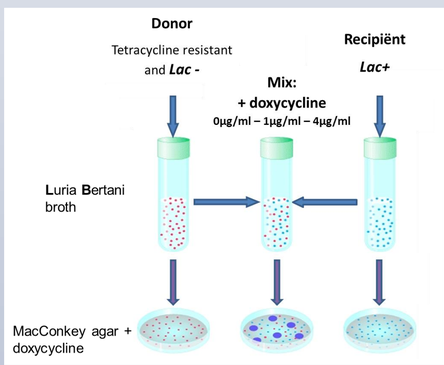
Donor strains carrying resistance plasmids:

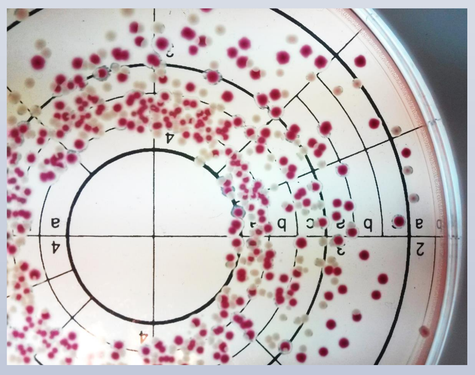
- Three commensal DOX resistant *E. coli* strains isolated from pigs
- Resistance genes and Inc groups: PCR
- Non-lactose fermenting mutants selected

Recipient strain:

- One commensal *E. coli* strain isolated from pigs
- Susceptible to all tested antibiotics
- Negative for plasmids of the same Inc groups as donor strains

6. *In vitro* bacterial competition experiments





Mix of 1 **donor** (D) + 1 **recipient** (R):

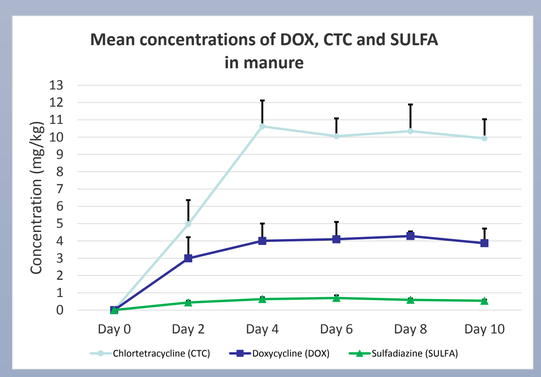
- **Ratio D/R:** 1/100 and 1/1000
- **Medium** (LB): blank - 1µg/ml DOX – 4µg/ml DOX
- **Incubation time** (37°C, shaking): 24h / 48h

Plating and **counting** white en red colonies on:

- MacConkey agar (MC): red colonies (= total recipients)
- MC + DOX: red colonies (= transconjugants) and white colonies (= donors)

Results

Antimicrobial concentrations in manure



Mean concentrations (+/- SD) of each antimicrobial rose to a steady state on day 4 of:

- 4 mg/kg wet weight (w.w.) **(DOX)**
- 10 mg/kg w.w. for **(CTC)**
- 500-700 µg/kg w.w. for **(SULFA)**
- **Trimethoprim**: all values were below the limit of detection, no quantification

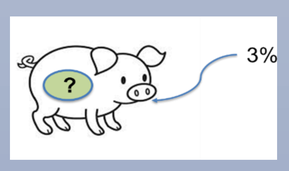
Antimicrobial concentrations in caecum and colon content

- **Tetracycline** concentrations are relatively **high** in general and highest concentrations are found in end colon
- **Sulfadiazine** concentrations are relatively **low** in general and highest concentrations are found in middle colon
- **Trimethoprim**: all values were below the limit of detection, no quantification

Transfer ratios (TR)

TR manure = $\frac{\text{Mean concentration manure (*)}}{\text{Mean concentration in feed}}$ (*) day 2 – day 10 of experimental period

TR caecum and colon content = $\frac{\text{Mean concentration caecum and colon content}}{\text{Mean concentration in feed}}$



Transfer ratio (TR) of antimicrobials compared to their oral bioavailability (BA) in pigs

Antimicrobial	TR Caecum / colon content	TR Manure	BA
Chlortetracycline	82%	92%	6%
Doxycycline	39%	52%	20%
Sulfadiazine	4.1%	4.5%	80-100%
Trimethoprim	ND	ND	60-80%

Effect of low concentrations of doxycycline (1 µg/ml and 4µg/ml) on resistance selection and transfer in *E. coli*

- Supplementation of the medium, both with **1 µg/ml** as with **4 µg/ml doxycycline**, resulted in a **strong selection of the resistant donor strain** compared to the blank medium.
- Counting of transconjugants in the supplemented media was not possible in most cases, due to the low number of transconjugants compared to the donor strain. Consequently, plasmid transfer rates could not be determined. However, in contrast with what could be expected, analysis of these results indicates that **plasmid transfer rates** for the selected strains are in most cases **lower in the supplemented media** compared to the blank medium.
- Further research is needed to quantify plasmid transfer rates (f.e. with rifampicin resistant recipient strain)

Characterization of selected doxycycline resistant strains

Strain n°	Phenotypic resistance profile	*tet* gene	Plasmid Inc group
1	AMP-SMX-STR-TET-TMP	*tetA*	Inc I1
2	AMP-CHL-STR-TET	*tetA*	Inc FrepB
3	SMX-STR-TET-TMP	*tetA*	Inc FrepB

AMP, ampicillin; CHL, chloramphenicol; SMX, sulphonamides; STR, streptomycin; TET, tetracycline; TMP, trimethoprim

Ratio D/R: 1/100		24h incubation			48h incubation		
		Blank	DOX 1µg/ml	DOX 4µg/ml	Blank	DOX 1µg/ml	DOX 4µg/ml
Donor 1	Total recipients (**R**) cfu/ml	1,43E+09	1,42E+06	o	1,29E+09	8,33E+04	1,17E+06
	Donors (**D**) cfu/ml	2,64E+07	1,40E+09	o	1,10E+08	1,17E+09	1,15E+08
	Transconjugants (**T**) cfu/ml	1,75E+06	x	o	1,09E+08	x	x
	Ratio donor/recipient (**D/R**)	1,85E-02	9,88E+02		8,54E-02	1,40E+04	9,86E+01
	Plasmid transfer rate (**T/R**)	1,23E-03			8,48E-02		
Donor 2	Total recipients (**R**) cfu/ml	1,38E+09	8,33E+04	8,67E+06	2,14E+09	x	2,42E+06
	Donors (**D**) cfu/ml	4,03E+07	1,18E+09	3,88E+08	8,33E+07	3,73E+08	2,18E+08
	Transconjugants (**T**) cfu/ml	4,44E+07	x	x	1,08E+08	x	x
	Ratio donor/recipient (**D/R**)	2,93E-02	1,42E+04	4,48E+01	3,90E-02		9,03E+01
	Plasmid transfer rate (**T/R**)	3,23E-02			5,03E-02		
Donor 3	Total recipients (**R**) cfu/ml	1,41E+09	1,09E+07	1,23E+07	1,22E+09	1,75E+06	2,33E+06
	Donors (**D**) cfu/ml	3,38E+07	5,54E+08	6,72E+08	8,33E+07	5,88E+08	1,78E+08
	Transconjugants (**T**) cfu/ml	1,34E+07	4,17E+05	5,83E+05	1,63E+08	1,67E+05	x
	Ratio donor/recipient (**D/R**)	2,40E-02	5,08E+01	5,48E+01	6,84E-02	3,36E+02	7,61E+01
	Plasmid transfer rate (**T/R**)	9,53E-03	3,82E-02	4,76E-02	1,33E-01	9,52E-02	

Ratio D/R: 1/1000		24h incubation			48h incubation		
		Blank	DOX 1µg/ml	DOX 4µg/ml	Blank	DOX 1µg/ml	DOX 4µg/ml
Donor 1	Total recipients (**R**) cfu/ml	o	3,38E+07	4,28E+07	1,35E+09	1,98E+07	3,83E+07
	Donors (**D**) cfu/ml	o	2,76E+08	2,38E+08	1,02E+07	4,56E+08	2,44E+08
	Transconjugants (**T**) cfu/ml	o	x	x	9,75E+06	x	x
	Ratio donor/recipient (**D/R**)		8,15E+00	5,54E+00	7,51E-03	2,31E+01	6,37E+00
	Plasmid transfer rate (**T/R**)				7,20E-03		
Donor 2	Total recipients (**R**) cfu/ml	9,03E+08	3,08E+07	3,48E+07	1,31E+09	2,29E+07	2,73E+07
	Donors (**D**) cfu/ml	4,72E+07	3,44E+08	2,44E+08	5,42E+07	3,58E+08	1,90E+08
	Transconjugants (**T**) cfu/ml	4,15E+07	x	x	1,85E+08	x	x
	Ratio donor/recipient (**D/R**)	5,22E-02	1,12E+01	7,03E+00	4,14E-02	1,56E+01	6,97E+00
	Plasmid transfer rate (**T/R**)	4,60E-02			1,42E-01		
Donor 3	Total recipients (**R**) cfu/ml	5,76E+08	1,51E+08	3,67E+07	8,30E+08	1,32E+08	5,58E+07
	Donors (**D**) cfu/ml	1,37E+07	3,90E+08	2,92E+08	3,08E+07	6,27E+08	3,81E+08
	Transconjugants (**T**) cfu/ml	3,23E+07	1,67E+05	x	1,87E+08	2,50E+05	x
	Ratio donor/recipient (**D/R**)	2,37E-02	2,59E+00	7,95E+00	3,71E-02	4,76E+00	6,82E+00
	Plasmid transfer rate (**T/R**)	5,60E-02	1,10E-03		2,25E-01	1,90E-03	

D, resistant donor strain (cfu/ml); R, recipient strain (cfu/ml); T, transconjugants (cfu/ml). Ratio D/R: ratio of donor and recipient strain (overnight culture volume) at the start of incubation. Blank: non-supplemented Luria Bertani broth.
DOX 1 µg/ml: LB broth + doxycycline 1µg/ml DOX 4 µg/ml: LB broth + doxycycline 4 µg/ml. x, counting impossible due to low number compared to the other strain. o, inconsistent results, need to be repeated.

Conclusions

The results of the animal experiment show that the poor oral bioavailabilities of tetracyclines may result in rather high concentrations in caecum, colon and manure, even at 3% cross-contamination of the feed. As expected, the high oral bioavailabilities of sulfadiazine and trimethoprim appear to result in very low gut concentrations. The *in vitro* research on the effect of 1 µg/ml and 4µg/ml doxycycline showed that both concentrations have a clear selective effect on the resistant donor strain. This effect seems higher at 1 µg/ml compared to 4 µg/ml, which could possibly be due to the fact that 4 µg/ml is the ECOFF value (Eucast) for doxycycline in *E. coli*. In contrast to what one would expect, plasmid transfer rates appeared lower in supplemented media compared to the blank medium. Further research is needed to quantify these transfer rates.

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